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# Colchicine, trifluralin, and oryzalin promoted development of somatic embryos in *Ilex paraguariensis (Aquifoliaceae)*

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**Summary**

A protocol for somatic embryogenesis and plant regeneration of *Ilex paraguariensis* St. Hil. from embryos cultures was developed. Heart stage zygotic embryos were removed from seeds of immature, light green fruit and treated with antimicrotubule agents (0.1; 0.2, and 0.5% colchicine for 24 and 48 h; 1; 10, and 20 $\mu$M of either trifluralin, $\alpha\alpha\alpha$- trifluoro- 2,6-dinitro-N,N- dipropyl-p-toluidine, or oryzalin, 3,5-dinitro-$N_4$, N-dipropylsulphate during 48 h). The embryos were cultured aseptically on quarter-strength Murashige and Skoog medium containing 3% sucrose, 0.65% agar (1/4MS), and 0.46 $\mu$M zeatin. Cultures were incubated in darkness at 27 $\pm$ 2 °C. All the treatments provoked a diminution of the number of germinated embryos and in some of the treated embryos somatic embryogenesis was induced. Somatic embryo maturation and conversion into whole plants could be achieved by culturing the embryos on 1/4MS lacking hormones and incubated at 27 $\pm$ 2 °C, 14 h photoperiod (116 $\mu$mol $m^{-2}s^{-1}$). Most of the plants regenerated from somatic embryos appeared morphologically normal and grew under greenhouse conditions. Only 2 plants out of 152 studied contained the tetraploid number of the chromosomes (2n = 4x = 80), meanwhile the rest of the plants had the normal diploid number of chromosomes (2n = 2x = 40). Somatic embryos with abnormal morphology were also observed.

## Introduction

'Maté' or 'Yerba Mate' (*Ilex paraguariensis* St. Hil.) is a South American tree belonging to the *Aquifoliaceae* family. The leaves are used to prepare a stimulating beverage very appreciated in some regions of Uruguay, Argentina, Brazil and Paraguay (Giberti, 1995).

Plant tissue culture of *I. paraguariensis* permits plant regeneration through *in vitro* culture of both, nodal segments (Rey & Mroginski, 1988; Sansberro et al., 1999) and immature embryos (Sansberro et al., 1998). However, plant regeneration via somatic embryogenesis han not been accomplished.

Somatic embryogenesis is important to the genetic improvement of plants using biotechnology for two major reasons. First, this system offers the capability to produce unlimited propagules and second, the embryogenic system could be used for genetic transformation. The importance of medium composition, especially growth regulators, in callus induction and plant regeneration through somatic embryogenesis has been reported in many plant species (Ammirato, 1989; Dunstan et al., 1995).

Treatments with some antimicrotubule agents, such as colchicine, trifluralin and oryzalin have been widely employed in order to induce chromosome doubling of several plant species by using various *in vitro* systems such as, anther (Alemanno & Guiderdoni, 1994; Redha et al., 1998), isolated microspore (Hansen & Andersen, 1998a,b; Zhao et al., 1996), ovule (Hansen et al., 1998), callus (Song et al., 1997) and cell suspension cultures (Hunault, 1997). In some

cases, colchicine treatments promoted somatic embryo formation by *in vitro* culture of anthers or isolated microspores (Sefc et al., 1997; Iqbal et al., 1994; Zaki & Dickinson, 1995).

The present paper reports the effect of colchicine, trifluralin and oryzalin on induction of somatic embryogenesis and plant production from immature zygotic embryos of *Ilex paraguariensis* cultured *in vitro*.

## Materials and methods

Immature green fruits from greenhouse-grown plants of *Ilex paraguariensis* St. Hil, gently provided by Establecimiento Las Marías (Virasoro, Corrientes, Argentina), were harvested in January 1999. One sample of fruits was harvested and immediately used for embryo excision, while another sample of fruits was previously stored in a refrigerator (4 °C) for 15 days. Fruits were surface sterilized in 70% ethanol for 5 min followed by immersion for 40 min in 2.7% (w/v) sodium hypochlorite, with two drops of Tween 20®. Subsequently, they were washed three times with autoclaved distilled water and kept in sterile water until excision of embryos under a stereomicroscope in a laminar flow hood.

The fruits were dissected under sterile conditions and the immature seeds were removed from each fruit. Each seed was also dissected and embryos at 0.25 mm in length at the heart-shaped stage with a portion of endosperm were removed and treated by immersion in 0.1, 0.2 or 0.5% colchicine for 24 or 48 h. The same explants were also immersed for 48 h in 1, 10 or 20 $\mu$M trifluralin ($\alpha\alpha\alpha$- trifluoro- 2,6-dinitro-N, N-dipropyl-p-toluidine) or in the same concentrations of oryzalin (3,5- dinitro-$N_4$, N-dipropylsulphate).

The solutions of colchicine, trifluralin and oryzalin were previously filter-sterilized (0.22 $\mu$m pore site) and all the treatments were done in darkness at 27 ± 2 °C on a gyratory shaker (100 rpm). After these treatments, the embryos were cultured on 3 ml of culture medium in 11-ml glass tubes (one embryo/ tube). The tubes were sealed with Resinite AF 50® (Casco S.A.I.C. Company, Bs.As. Argentina) and the cultures were grown in darkness at 27 ± 2 °C. There were 8–10 replicates for each treatment, and the experiments were repeated four times. After 30 days, the number of germinated embryos as well as the number of the cultures forming somatic embryos were recorded. The mean and standard error for each treatment were calculated.

The culture medium was that reported by Sansberro et al. (1998). It consisted of quarter-strength Murashige and Skoog (1962) medium containing 3% sucrose, 0.65% Sigma agar (A-1296) and 0.46 $\mu$M zeatin. The pH of the medium was adjusted to 5.8 with either KOH or HCl prior the addition of agar. Tubes were covered with aluminum foil and autoclaved at 1.46 kg/cm$^2$ for 20 min.

Somatic embryos at the cotyledonary stage or small clusters of embryos were isolated and transferred to culture tubes containing quarter-strength Murashige and Skoog (1962) medium with 3% sucrose, 0.65% agar without growth regulators to promote plantlet development. The cultures were maintained at 27 ± 2 °C, with a 14 h photoperiod (116 $\mu$mol m$^{-2}$s$^{-1}$ from fluorescent lamps).

Plantlets bearing two true leaves derived from both germination of zygotic embryos or from conversion of somatic embryos were transplanted successfully out of aseptic tubes to pots containing vermiculite and were maintained in a growth room at 27 ± 2 °C with a 14 h photoperiod (116 $\mu$mol m$^{-2}$s$^{-1}$). Relative humidity was maintained at 95–100% during the first week and then decreased gradually until establishment in a greenhouse.

For chromosome counting, root tip meristems from plantlets were collected, pretreated with 0.002 M 8-hydroxiquinoline at room temperature for 3 h followed by fixation at 4 °C for 24 h in a 5:1 ethanol-lactic acid mixture. Subsequently, the root tips were stored at 4 °C in 70% ethanol until the cytological analysis. For chromosome staining, roots were hydrolyzed in 1 N HCl at 60 °C for 8 min, stained using the Feulgen technique, macerated in a drop of 3% acetic orcein, and then squashed on a slide. Observations of metaphase chromosomes were made to determine the ploidy levels of the plants. At least 20 figures were observed for each slide.

## Results and discussion

Earlier we have reported that when heart stage zygotic embryos of *Ilex paraguariensis* were removed from seeds and cultured on an appropriate medium, they germinated and developed into complete plants (Sansberro et al., 1998). During the present investigations, treatment of those embryos with antimicrotubule agents such as colchicine, trifluralin and oryzalin provoked a reduction of the number of embryos, which have germinated. The analysis of the results (Table 1)

*Table 1.* Somatic embryo induction on zygotic embryos of *Ilex paraguariensis* culture *in vitro* after different rates and exposure times to colchicine

| Source of explants | Colchine treatment | | % of zygotic embryos converted to normal seedling | % explants with somatic embryos | Mean number of somatic embryos/ explant* |
|---|---|---|---|---|---|
| | Concentration (%) | hours | | | |
| Fruits | 0 | 24 | 41(± 3) | 0 | 0 |
| without | 0.1 | 24 | 36(± 7) | 0 | 0 |
| cold | 0.2 | 24 | 41(± 5) | 0 | 0 |
| treatment | 0.5 | 24 | 30(± 4) | 20(± 7) | 6.2 |
| | 0 | 48 | 43(± 6) | 0 | 0 |
| | 0.1 | 48 | 29(± 4) | 5(± 3) | 5.3 |
| | 0.2 | 48 | 37(± 1) | 5(± 4) | 8.4 |
| | 0.5 | 48 | 25(± 1) | 2(± 2) | 2.1 |
| Fruits | 0 | 24 | 45(± 5) | 0 | 0 |
| with | 0.1 | 24 | 32(± 6) | 2(± 1) | 1.2 |
| cold | 0.2 | 24 | 34(± 2) | 0 | 0 |
| treatment | 0.5 | 24 | 27(± 2) | 10 | 3.4 |
| | 0 | 48 | 43(± 4) | 0 | 0 |
| | 0.1 | 48 | 21(± 2) | 12(± 3) | 6.8 |
| | 0.2 | 48 | 29(± 3) | 11(± 4) | 1.6 |
| | 0.5 | 48 | 27(± 5) | 8(± 2) | 3.2 |

* Only embryos at mature-stage were scored.

*Table 2.* Somatic embryos induction on zygotic embryos of *Ilex paraguariensis* cultured *in vitro* after treatment with three concentration of trifluralin

| Source of explants | Concentration of trifluralin treatment (in $\mu$M) | % of zygotic embryos converted to normal seedling | % explants with somatic embryos | number of somatic embryos/ explant* |
|---|---|---|---|---|
| Fruits without | 0 | 44(± 3) | 0 | 0 |
| cold treatment | 1 | 36(± 1) | 0 | 0 |
| | 10 | 12(± 4) | 8(± 1) | 0.9 |
| | 20 | 16(± 1) | 0 | 0 |
| Fruits with | 0 | 46(± 2) | 0 | 0 |
| cold treatment | 1 | 21(± 3) | 7(± 3) | 2.6 |
| | 10 | 14(± 4) | 0 | 0 |
| | 20 | 10(± 7) | 0 | 0 |

* Only embryos at mature-stage were scored.

revealed that various concentrations of colchicine (0.1, 0.2, and 0.5%) applied during both 24 and 48 h, affected the percentage of zygotic embryos converted to normal seedlings. This was increased when the zygotic embryos were treated for 48 h with trifluralin (Table 2) or with oryzalin (Table 3). In the last case, some concentrations prevented completely the germination of zygotic embryos. These results were independent of the source of the explants used (fruits with or without cold pretreatment). The reduction of the number of the plantlets obtained caused by highest antimicrotubule agent concentrations used in our study was expected and must be due to the strong toxic effect of colchicine in plant cells by destroying spindle fibers and modifying the differentiation process (Eigsti & Dustin, 1955) or because of the effect of the herbicide trifluralin and

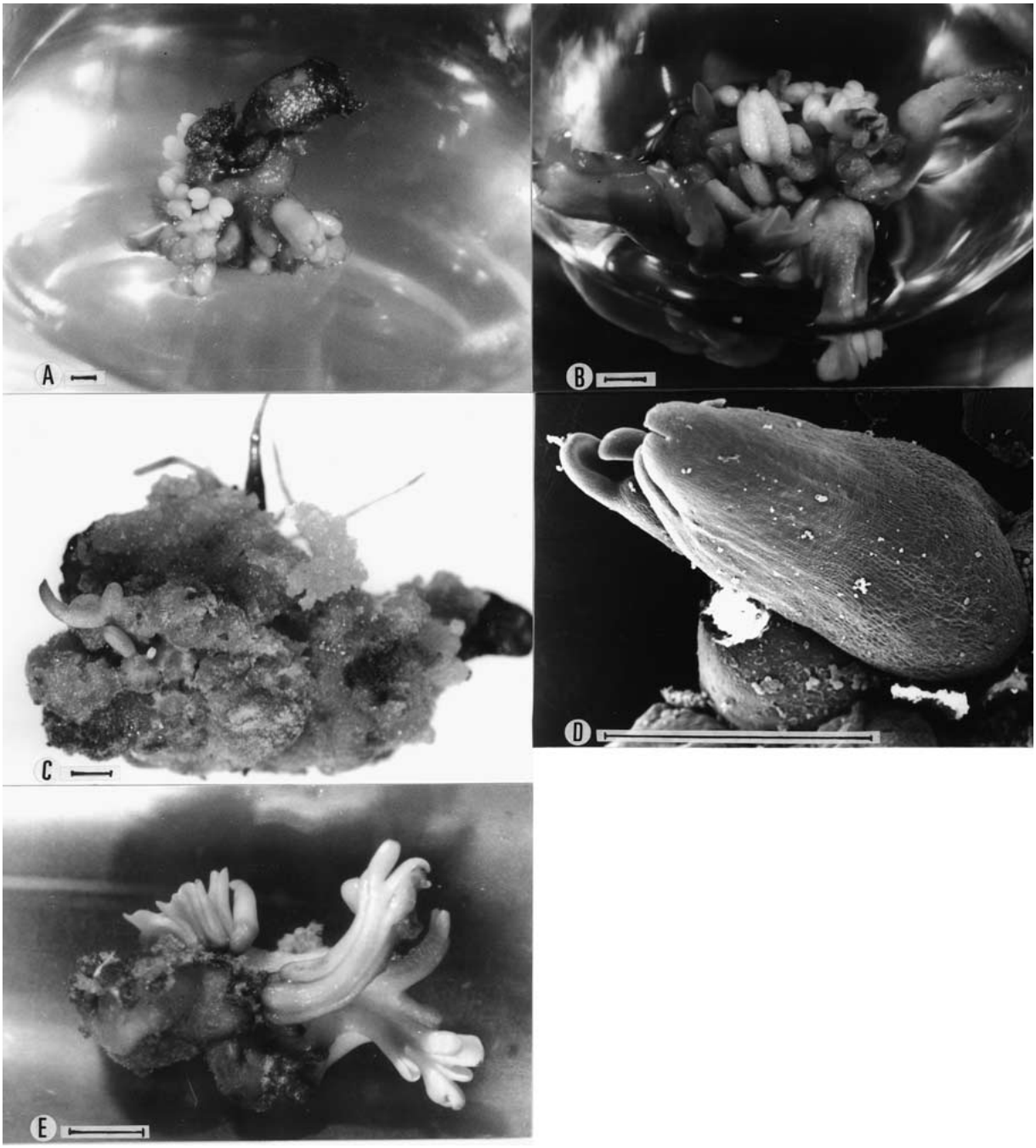


*Figure 1.* Somatic embryogenesis from zygotic embryos of *Ilex paraguariensis*. Bars represent 1 mm. A and B: direct somatic embryogenesis. C: indirect somatic embryogenesis. D: somatic embryo. E: Fused somatic embryos.

*Table 3.* Somatic embryos induction on zygotic embryos of *Ilex paraguariensis* cultured *in vitro* after treatment with three concentration of oryzalin

| Source of explants | Concentration of oryzalin treatment (in $\mu$M) | % of zygotic embryos converted to normal seedling | % explants with somatic embryos | number of somatic embryos/ explant* |
|---|---|---|---|---|
| Fruits without cold treatment | 0 | 40(± 4) | 0 | 0 |
| | 1 | 9(± 4) | 10(± 2) | 2.9 |
| | 10 | 0 | 0 | 0 |
| | 20 | 2(± 1) | 0 | 0 |
| Fruits with cold treatment | 0 | 37(± 6) | 0 | 0 |
| | 1 | 6(± 1) | 0 | 0 |
| | 10 | 0 | 0 | 0 |
| | 20 | 0 | 8(± 2) | 0.4 |

* Only embryos at mature-stage were scored.

oryzalin on the microtubule depolymerization (Bartels & Hilton, 1973; Hugdahl & Morejohn, 1993).

On the other hand, after 2–3 weeks culture in various treatments with colchicine, trifluralin or oryzalin a profuse differentiation of somatic embryos was observed. Embryogenesis can follow two routes: a) direct embryogenesis, where embryos are initiated directly on the surface of the explants from cells without callus formation (Figures 1A–B). The white somatic embryos contrasted with the brown original explants, and b) indirect embryogenesis, where embryos are formed from a friable callus (Figure 1C). Although, most of the somatic embryos formed a loose aggregate such that individual embryos could be separated easily (Figure 1D), in some cases fused embryos were formed (Figure 1E). The excised zygotic embryos, especially at immature stage, become an ideal source of explants for the development of embryogenic cultures in a large number of plant species (Tinsera et al., 1979; Monnier, 1995; Sharma et al., 1996) including *Ilex aquifolium*, where zygotic embryos excised at heart stage and cultured on Linsmaier & Skoog (1965) medium lacking hormones have permitted plant regeneration through the development of somatic embryos (Hu & Sussex, 1971) of several horticultural clones. The potential of somatic embryo production ranged from 8 to 18% of the cultures. Somatic embryos have also been observed in embryo cultures of *Ilex cornuta* and *I. opaca* (Hu et al., 1978; Hu, 1989). In these species, the culture conditions that support initiation and development of somatic embryos are very simple, and no exogenous source of growth regulators was required (Hu & Sussex, 1971). On the contrary in this study, the induction of somatic embryos of *Ilex paraguariensis* required, besides the antimicrotubule treatment, a cytokinin (Tables 1, 2, and 3). The results show that, in general, the percentage of explants that formed somatic embryos as well as the mean number of embryos/explant are not related with any treatment. The source of explants (fruits with or without cold storage) has no influence on the development of embryogenic cultures. Although colchicine may promote embryogenesis in either anther or microspore cultures (Zaki & Dickinson, 1991, 1995), there are no reports in the literature regarding use of colchicine to stimulate somatic embryogenesis in zygotic embryo cultures. It is not possible currently to find an explanation of this response, although it could be related to the induction of synthesis of stress proteins. Additionally, this is the first study to show that both trifluralin and oryzalin can be used to promote somatic embryogenesis in zygotic embryo cultures of *Ilex paraguariensis*.

Although the origin of the somatic embryos of *Ilex paraguariensis* has not been studied, it is interesting to note that they were initiated only of seedlings in which the shoot failed to develop. There is no embryo formation when the zygotic embryos grow into plantlets. The same behavior was described in *I. aquifolium* (Hu & Sussex, 1971), and in *Biota orientalis* (Konar & Oberoi, 1965). After 30–40 days of culture, somatic embryos isolated from small clusters of embryos were subcultured in light conditions on medium lacking growth regulators. After 30 days culture, most of the *Ilex paraguariensis* somatic embryos had a single axis with cotyledons and produced normal green plants,

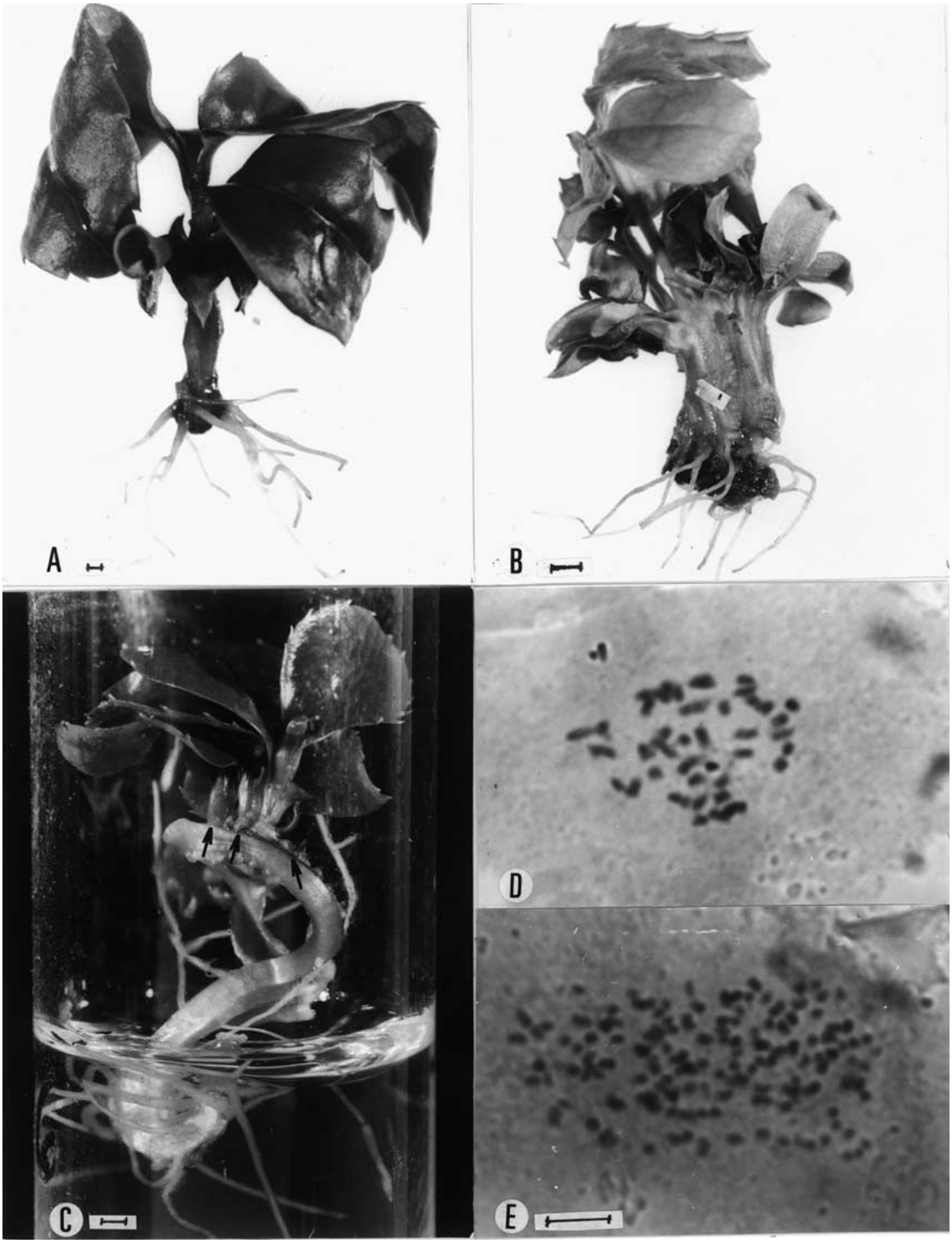


*Figure 2.* Somatic embryogenesis from zygotic embryos of *Ilex paraguariensis*. A: Normal plantlets derived by conversion of somatic embryos. The principal root was used for chromosome analysis. Bar represents 1 mm. B: fused plants. Bar represents 1 mm. C: Secondary embryogenesis on hypocotyl of plantlets. Arrow indicates secondary somatic embryos. Bar represents 1 mm. D: 2n = 2x = 40 chromosomes in root tip cell of plantlets regenerated from somatic embryos. Bar represents 5 $\mu$. E: 2n = 4x = 80 chromosomes in root tips cell of a plant regenerated from zygotic embryo. Bars represent 5 $\mu$.

*Table 4.* Chromosome numbers of *Ilex paraguariensis* obtained by culture of zygotic embryos after treatments with colchicine, trifluralin or oryzalin

| Treatment | Plantlets observed | | | | | |
|---|---|---|---|---|---|---|
| | derived from germination of zygotic embryos | | | derived from somatic embryos | | |
| | Total | diploid (2n = 40) | tetraploid (2n = 80) | Total | diploid (2n = 40) | tetraploid (2n = 80) |
| Colchicine (0.1 or 0.2% during 24 or 48 h) | 39 | 39 | 0 | 20 | 20 | 0 |
| Colchicine 0.5% during 48 h | 15 | 13 | 2 | 7 | 7 | 0 |
| Trifluralin (1 and 10 $\mu$M) | 31 | 31 | 0 | 8 | 8 | 0 |
| Oryzalin (20 $\mu$M) | 19 | 19 | 0 | 13 | 13 | 0 |

which are similar to that obtained through germination of seeds (Figure 2A). However, somatic embryos showing different morphologies also were observed. These embryos generally differed in axis configuration and degree of hypocotyl/epicotyl development. Most of these embryos failed to convert into plants and if converted, gave plantlets with various abnormalities, such as fused plantlets (Figures 2B). Some of them failed to exhibit both root and hypocotyl growth, with a profuse growth of leaves or, on the contrary, plantlets with poor development of the leaves. Some of the embryos had shown bifurcation. In some embryos treated with 10 $\mu$M trifluralin, secondary embryogenesis was observed on the hypocotyl of primary somatic embryos (Figure 2C). Several of these abnormalities have been described in other plant species such as peanut (Wetzstein & Baker, 1993), soybean (Bucheim et al., 1989), and *Populus* spp. (Michler & Bauer, 1991).

An overall conversion rate of about 50% was obtained with *Ilex paraguariensis* somatic embryos, which have exhibited a normal morphology. The resulting plantlets were transplanted into pots. The survival rates of these plants was almost 90% and potted plants grew normally under greenhouse conditions. One hundred and fifty two of them (104 by germination of zygotic embryos and 48 from somatic embryos) were randomly chosen for chromosome analysis. The results are presented in Table 4. All the control plantlets, as well as all the plantlets derived from embryos treated with oryzalin and trifluralin, had 2n = 2x = 40 chromosomes (Figure 3D). Only two plantlets derived from colchicine-treated embryos were tetraploids (Figure 3E). Unfortunately these plants did not survive in the greenhouse.

In conclusion, these studies demonstrate that: 1) only a very low frequency of tetraploid plants of *Ilex paraguariensis* were induced when zygotic embryos were treated with 0.5% colchicine for 48 h, and then cultured *in vitro*. The herbicide trifluralin and oryzalin have shown to be totally ineffective for doubling of chromosome number. 2) All the antimicrotubule agents tested promoted somatic embryo differentiation. However, further research is needed to increase of frequency of conversion of somatic embryos in whole plants.

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